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Use of B7 costimulatory molecules as adjuvants in a prime-boost vaccination against Visna/Maedi ovine lentivirus

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Abstract

RNA transcripts of the B7 family molecule (CD80) are diminished in blood leukocytes from animals clinically affected with Visna/Maedi virus (VMV) infection. This work investigates whether the use of B7 genes enhances immune responses and protection in immunization-challenge approaches. Sheep were primed by particle-mediated epidermal bombardment with VMV gag and env gene recombinant plasmids together with plasmids encoding both CD80 and CD86 or CD80 alone, boosted with gag and env gene recombinant modified vaccinia Ankara virus and challenged intratracheally with VMV. Immunization in the presence of one or both of the B7 genes resulted in CD4⁺ T cell activation and antibody production (before and after challenge, respectively), but only immunization with CD80 and CD86 genes together, and not CD80 alone, resulted in a reduced number of infected animals and increased early transient cytotoxic T lymphocytes (CTL) responses. Post-mortem analysis showed an immune activation of lymphoid tissue in challenge-target organs in those animals that had received B7 genes compared to unvaccinated animals. Thus, the inclusion of B7 genes helped to enhance early cellular responses and protection (diminished proportion of infected animals) against VMV infection.

Keywords Sheep; Small ruminant lentivirus; DNA vaccination; B7 costimulatory molecules

1. Introduction

Small ruminant lentiviruses (SRLV) include Visna/Maedi Virus (VMV) and caprine arthritis-encephalitis virus (CAEV). SRLV infect the monocyte/macrophage lineage in sheep and goats and cause chronic inflammatory lesions in the lung, carpal joints, mammary glands, and brain in these ruminant species [1]. SRLV are widely spread and strategies based on early diagnosis, management and culling of seropositive animals have been

applied in order to eradicate infection. In natural SRLV infections of small ruminants, the response of the immune system to virus in tissues may lead to pathology [2], [3], [4], [5] and [6], which may increase in heavily infected animals [2], [7] and [8] till the animal's death. Although vaccination may prevent infection, minimize clinical symptoms or delay the onset of disease resulting in an improvement of animal welfare and avoidance of production losses (reviewed in Ref. [9]), efficient immunoprophylactic tools have not been developed against SRLV and immunization has not conferred sterilizing immunity so far.

DNA vaccination is an alternative approach to conventional vaccines, triggering both antibody and cell-mediated immune responses [10]. In goats, SRLV immunization with env plasmids has led to decreased viral replication and load and diminished disease [11] whilst gag gene or peptide immunization has resulted in enhancement of proviral load or disease [12] and [13]. In sheep, immunization may lead to protection against early lesion development or against viral infection (decreased load) [14], [15] and [16]. Vaccination with inactivated virus leads to disease [12], [17] and [18], but using an attenuated VMV clone results in a diminished number of viral isolations after challenge [19]. Mucosal immunization using plasmids containing VMV env confers a protective effect seen by decreased proviral load [14] and [16], whilst the use of the gag gene enhances protection against early lesion development [16]. When the same plasmids are delivered intradermally and this is followed by modified vaccinia Ankara virus (MVA) boosting, only the gag gene (or gag in combination with env) gives rise to partial protection against infection [15], but when the vaccination protocol is changed [20], pre-existing immune responses to GAG proteins do not prevent infection. Thus, immunization routes and viral genes used clearly affect the results of vaccination.

One of the limiting factors in DNA vaccination is antigen presentation after host-cell transfection with exogenous DNA [21]. Full activation of T cells requires both an antigen-specific stimulus provided by MHC-peptide complex and a costimulatory signal [22] and [23]. The engagement of CD28 on the surface of T cells by the costimulatory B7-1 (CD80) [24] and [25] or B7-2 (CD86) [26] and [27] molecules expressed by antigen presenting cells (APCs) provides a potent costimulatory signal, leading to T cell proliferation, differentiation, and cytokine production. However, CD80 and CD86 have higher affinity for the T cell inhibitory receptor CTLA-4 than for CD28 [28] and [29]. This receptor is up-regulated after T cell activation and it is suggested that these interactions are important in helping to curb T cell responses. CD86 exhibits faster dissociation kinetics than CD80 in interactions with both CD28 and CTLA-4 [29]. Also CD86 is constitutively expressed on B cells, monocytes and dendritic cells whilst CD80 is inducible [23] and [26]. Therefore, CD80 and CD86 appear to have functional differences in T cell activation.

Ovine CD80 [30] and CD86 [31] genes have been identified recently. The mRNA levels of these molecules in peripheral blood mononuclear cells (PBMC) have been studied at different stages of SRLV infection [32], being increased in VMV-infected asymptomatic sheep and associated with positive VMV-specific T cell proliferative responses. In contrast, low levels of these molecules (especially of CD80) are found in clinically affected sheep, being associated with impairment of antigen-specific cellular recall responses.

This study investigates the immune responses and degree of protection, against viral infection (load) and/or immunity-related lesion development, obtained by particle-mediated epidermal delivery (PMED) immunization of sheep against VMV, priming with plasmids encoding VMV (gag and env) genes together with plasmids encoding B7 genes (CD80 and CD86) or CD80 gene alone, and using a recombinant MVA for booster immunization.

2. Materials and methods

2.1. Animals

Thirty castrated one-year-old males from a VMV-free certified flock belonging to the Lleyn sheep breed from the United Kingdom were used to avoid possible breed effects in comparisons with previous studies involving similar immunization schemes. Absence of VMV infection was confirmed by a commercial ELISA (Elitest MVV/CAEV, Hyphen Biomed) and PCR (as described below). The animal experiments were performed following national regulations and institutional guidelines.

2.2. Plasmids and recombinant modified vaccinia virus Ankara used for immunization

Plasmids used for immunizations were derivatives of pN3, generated from pEGFP-N3 (Clontech Laboratories Inc.) by removing the EGFP gene, and which contains the eukaryotic HCMV immediate early promoter to drive expression of the gene of interest. pN3 was used as the control empty plasmid in immunizations and also to make pN3-gag and pN3-env, which encode the VMV gag p55 and env gp150 genes (EV1 strain, [33] and [34]) previously used in other vaccination studies [15] and [16]. Large-scale preparations of endotoxin-free plasmid DNA were made using a commercial maxi-prep kit (Qiagen Ltd.) according to the manufacturer's instructions. Expression of p55 GAG and gp150 ENV was determined by RT-PCR and Western blotting (WB) [16] and [34].

Ovine CD80 (AY390555) and CD86 (AY491977) cDNA, cloned into pGEM-T easy plasmids [30] and [31], were sub-cloned into the eukaryotic expression plasmid pN3 to produce pN3-CD80 and pN3-CD86, respectively. Expression of pN3-CD80 and pN3-CD86 was assessed by specific mRNA transcription in transfected ovine skin fibroblasts using RT-PCR using the following primers: forward SG80sFW 5'-CAT CAC CCC AAA GAG CGT G-3' and reverse B7-1RV 5'-TGG AAA ACC TCC AGA GG-3' to amplify a 415 nucleotide (nt) region of the ovine CD80 cDNA; and forward CD86-TM-FW 5'-GAC AAT CTT CTG TGT CCT GCA ACT TGA GCC-3' and reverse CD86-2RV 5'-TCC AGG TTT TTG GAG TTC TAC CC-3' to amplify a 110 nt region of the ovine CD86 cDNA.

Plasmid transfection was performed with Lipofectamine 2000 (Invitrogen) in monolayers of ovine skin fibroblasts, as specified by the manufacturer. Transfected cells were incubated for 48 h and total RNA extracted and analyzed by RT-PCR for the presence of CD80 or CD86 RNA. Using the appropriate controls (e.g. no RT, empty vector transfected cells) both the CD80 and CD86 expression plasmids were shown to express the appropriate RNA (data not shown). MVA virus expressing either the gag p55 (MVA-gag) or the env gp150 (MVA-env) genes of VMV EV1 were used for immunization as described previously [16] and [34]. For the control group, immunization with recombinant MVA expressing β -galactosidase (MVA-pSC11) was used. Viral protein expression was determined by WB using anti-GAG and anti-ENV specific rabbit sera [34].

2.3. Preparation of DNA-coated gold particles for gene gun immunization

Gene gun parameters used for immunization were as previously described [15] and [35]. Briefly, DNA plasmids were precipitated onto gold particles (BioRad Laboratories Ltd.) resulting in a DNA loading ratio of 4 μ g/mg of gold. The mixture was allowed to precipitate, washed in dehydrated 99.9% ethanol (VWR International Ltd.) and then re-suspended in ethanol containing polyvinylpyrrolidone. Each cartridge contained approximately 1 μ g of plasmid DNA. Delivery was performed intradermally using the Helios Gene gun system (BioRad Laboratories Ltd.). The gene gun discharge pressure used to propel the DNA-coated gold particles into the dermal-epidermal junction was 435 psi.

2.4. Expression and purification of recombinant GAG proteins and mock protein preparations

Plasmids (pRSET) containing VMV strain EV1 gag p14, p17 and p25 [36] were used once sequences were verified. Recombinant p14, p17, p25 and mock preparations were produced as described previously [16].

2.5. Immunization and challenge

Table 1 shows the immunization groups and number of animals used. All groups received a total of 30 µg of DNA per animal by gene gun at weeks 0 and 4 (10 µg of each plasmid except the gag–env–CD80–CD86 group that received 10 µg of each VMV plasmid and 5 µg each of the CD80 and CD86 plasmids). The same sheep were boosted subcutaneously at week 10 with recombinant MVA-gag and MVA-env (108 pfu each). The control group received MVA-pSC11 (2 × 10⁸ pfu).

Table 1.

Table 1.

Inoculum received by the different study groups.

Animal group	Number of animals	Inoculum	
		Plasmid prime and boost ^a (weeks 0, 4)	MVA boost ^b (week 10)
Control	7	pN3 (30 µg)	MVA-pSC11
<i>gag–env</i>	7	pN3- <i>gag</i> (10 µg) + pN3- <i>env</i> (10 µg) + pN3 (10 µg)	MVA- <i>gag</i> + MVA- <i>env</i>
<i>gag–env–CD80</i>	8	pN3- <i>gag</i> (10 µg) + pN3- <i>env</i> (10 µg) + pN3- <i>CD80</i> (10 µg)	MVA- <i>gag</i> + MVA- <i>env</i>
<i>gag–env–CD80–CD86</i>	8	pN3- <i>gag</i> (10 µg) + pN3- <i>env</i> (10 µg) + pN3- <i>CD80</i> (5 µg) + pN3- <i>CD86</i> (5 µg)	MVA- <i>gag</i> + MVA- <i>env</i>

a Particle-mediated epidermal delivery, a total of 30 µg DNA per animal.

b Subcutaneous; 2 × 10⁸ pfu of modified vaccinia Ankara virus (MVA) (10⁸ per recombinant vaccinia) per animal.

VMV strain EV1 [33] grown and titrated as described previously [37] was used to challenge sheep with 1 × 10³ TCID₅₀ in 1 ml of PBS 12 weeks after priming via the intratracheal route, as described elsewhere [38].

2.6. Sampling

Blood samples were collected before priming (hereafter called week 0), after DNA priming (week 7), after recombinant MVA boosting but before challenge (week 12), and after VMV challenge at different time points (weeks 16, 20 and 24, that is weeks 4, 8 and 12 post-challenge). Sheep were euthanised and necropsied 25 weeks after the start of the experiment (13 weeks post-challenge). Lung lobes and mediastinal lymph node (MLN) samples were taken as previously described [15] and [16].

2.7. Measurement of anti-VMV antibodies

The presence of VMV-specific antibodies was assessed in serum after immunization and challenge using a commercial VMV antibody ELISA test (Elitest®, from Hyphen Biomed, Neuville-sur Oise, France) that employs as plate coating VMV antigens a synthetic peptide of the ENV transmembrane protein (TM) and recombinant GAG p25 protein [39]. Samples were analyzed in duplicate. The ELISA score (optical density, O.D. ratio) was calculated as the ratio between the mean absorbance (A₄₅₀) of a sample and the mean absorbance of the kit controls (cut-off), according to the manufacturer's instructions. ELISA scores ≥1 were considered positive.

An ELISA method using whole virus for coating (140 ng per well) was applied as previously described [16]. The positivity threshold was established for each plate as the mean O.D. value of known negative serum samples plus two times the standard deviation (mean + 2S.D.). Dilution series of sera were started at 1 in 100. The reciprocal of the last positive serum dilution was taken as the antibody titre and results were expressed as a box plot which includes the median value and the interquartile range.

Neutralizing antibody (NtAb) determinations were performed in six replicates (100 TCID₅₀ virus per well) as previously described [19] except that the incubation of the virus with serum at the different dilutions was done for 36 h and the cells used for the assay were monolayers of GSM-T β -gal cultures (kindly supplied by Dr. Valas, AFSSA, Niort, France). These cells were incubated with the mixture for 10 days, fixed and stained with X-gal. GSM-T β -gal cells are goat synovial membrane cells transfected and permanently expressing a β -galactosidase construct that contains a Rev Responsive Element (RRE) so that β -galactosidase protein is only expressed if Rev protein of the virus is present [40]. Thus, the presence of the virus in the supernatant was determined by blue staining due to the presence of the REV protein. NtAb titres of sera were calculated as the reciprocal of the serum dilution that caused loss of infectivity in 50% of inoculated cultures. Control cultures were inoculated with virus in the absence of antiserum.

To detect antibody by WB, virus antigen was diluted with 125 μ l of lysis buffer (0.05 M Tris pH 7.2, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethyl-sulfonyl fluoride) and 125 μ l of loading buffer (0.5 M Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.1 M dithiothreitol, bromophenol blue), applied to a 4% stacking gel and a 12% gradient separation gel and blotted onto a nitrocellulose transfer membrane after electrophoresis. Transfer was carried out at 4 °C for 75 min in 0.25 M Tris, 1.92 M glycine, 20% methanol and 0.01% SDS, at 100 V and 250 mA. After transfer, the membrane was blocked for at least 1 h at 37 °C with PBS containing 0.2% Tween 20 (PBS-T) and 3% bovine serum albumin (BSA). After blocking, the membrane was cut into strips and incubated with serum samples diluted 1:100 for 1 h at 37 °C with shaking. Rabbit anti-sheep IgG conjugated to peroxidase was used at a dilution of 1:2000 in PBS-T with 1% BSA for 1 h at 37 °C and reactions were developed using Chemiluminescence (Amersham ECL Western blotting detection reagents, GE Healthcare).

2.8. Measurement of anti-VMV T cell reactivity

Heparinized blood was collected (10 U/ml final) and PBMC isolated on a Ficoll-Hypaque gradient ($\delta = 1.077$; Lymphoprep, Axis-Shield), then suspended in RPMI-1640 medium with 25 mM HEPES (Sigma–Aldrich Company Ltd.), supplemented with 2 mM L-glutamine, 50 μ M beta-mercaptoethanol, 100 U penicillin and 100 μ g streptomycin/ml, 2.5 μ g amphotericin B/ml and 10% foetal calf serum (FCS) (10%RPMI).

A cytotoxic assay was performed for the detection of precursor cytotoxic T lymphocytes (CTL). Animals were deemed to show the presence of CTL if the percentage specific ⁵¹Cr release from VMV infected minus mock infected autologous cells was >10% above that from heterologous infected minus mock infected cells [16], in which the killing of mock infected and heterologous cells was less than 10%. The frequency of CTL-positive animals was used for analysis of results. VMV GAG-specific T cell proliferative and CTL responses were compared within and between groups throughout the experimental period using PBMC.

T cell proliferation assays were performed in quadruplicate as described elsewhere [16]. Briefly, 10⁵ PBMC in 10% RPMI were mixed with different dilutions of recombinant GAG proteins (from 50 μ g/ml to 6.25 μ g/ml for p25 and p14; and from 25 μ g/ml to 3.12 μ g/ml for p17). Positive Concanavalin A (ConA at 5 μ g/ml) and negative (mock antigen) controls were included. Cells were labelled with [³H] thymidine (0.037 MBq/well, GE Healthcare) and cellular proliferation assessed by measuring the incorporation of [³H] into

the cells (cpm). The stimulation index (S.I.) was calculated for each antigen using the formula $S.I. = \text{cpm with antigen} / \text{cpm with mock antigen}$. Results were analyzed using the median stimulation indices of all dilutions for each protein. An individual animal was considered to show positive T cell reactivity if the stimulation index was greater than 3.

2.9. Measurement of proviral load

2.9.1. Proviral load in blood

EDTA blood (8 ml) was collected at each time point and PBMC isolated on a Ficoll-Hypaque gradient ($\delta = 1.077$; Lymphoprep, Axis-Shield). Following erythrocyte lysis, genomic DNA was extracted with a QIAamp DNA Blood Mini Kit (Qiagen Ltd.) according to the manufacturer's instructions. DNA was quantified by spectrophotometry (Bio-Rad SmartSpec Plus Spectrophotometer, Bio-Rad) and a final concentration of 100 ng/ μ l was used. Five microliters of freshly prepared DNA were used in a 25 μ l real-time PCR assay specific for VMV gag p25. Gag amplicon length was 106 nt. Primers and probe sequences were as follows: sense primer S2: 5'-TCAACAGGCATCACAGGCTAATA-3' (nt. 1249–1271); antisense primer AS2: 5'-GTTACCTGGCCTATGCGACAT-3' (nt. 1334–1353); antisense probe: 5'-ACCGCTCTCAAGGCTGTTATGACCCA-3' (nt. 1301–1325). Nucleotide positions refer to the published EV1 strain sequence [33]. A dual-labelled probe (Operon) was used with 6-FAM as the 5' fluorophore and a 3' Black Hole Quencher (BHQ1a-Q). DNA samples were tested three times in triplicate (nine replicates in all). Ten-fold serial dilutions of a plasmid (pDRIVE, Qiagen Ltd.) carrying a 567 bp EV1 gag fragment (nt. 963–1529) were used to generate the standard curve. Plasmid copy numbers ranged from 10 to 106 per reaction. Positive and negative controls were included in each assay. Reactions were carried out in an ABI 7900 system (Applied Biosystems), with the following thermal profile: 10 min at 95 °C and then 45 cycles of 95 °C 15 s and 60 °C 1 min.

Mean copy number per reaction was converted to mean copy number per microgram of template DNA for each animal. Animals which showed positive proviral load in at least one time point of the three time points tested (weeks 16, 20 and 24) in the post-challenge period, were considered infected.

2.9.2. Proviral load in tissues

One hundred milligrams of tissue from MLN and lung sections as for the pathological evaluation (four lobes of the lung or the MLN, see below) were taken at necropsy and stored in RNA Later (Qiagen Ltd.) at –80 °C. Tissue samples were minced with a scalpel and homogenized with a pestle in 680 μ l of lysis buffer (5 M NaCl, 1 M Tris–HCl pH 8.5, 0.5 M EDTA, 10% SDS). Following incubation with proteinase K at 56 °C, DNA was extracted and the proviral copy number measured, as described above, from each lung piece and lymph node. Mean copy number per reaction was converted to mean copy number per microgram of template DNA for each animal. Animals were considered positive if the provirus was detected in at least one of the tissue sections analyzed.

2.10. Pathology studies

Lungs were removed aseptically and studied first macroscopically for pathological changes (colour, consistence, size and weight). Samples from four different lung lobes (the right accessory cranial lobe, the right apical medial lobe, the right caudal lobe and the left cardiac cranial lobe) and MLN were taken for histological studies. Tissues were fixed in 10% phosphate-buffered formalin (Sigma), embedded in paraffin and sectioned according to standard procedures. Sections were stained with Hematoxylin–Eosin and scored blind for pathology by two independent pathologists.

Histological changes in lung and MLN were scored: in the lung, lymphoid follicle hyperplasia and interstitial pneumonia as well as bronchial associated lymphoid tissue hyperplasia and perivascular infiltrates were scored on a scale of 0–3; and in the MLN, lymphoid follicle reactivity and cortical hyperplasia were scored on a scale of 0–1. The mean of all scores was used for each animal.

2.11. Statistical analysis

Fisher's exact test was used for comparisons on frequency of individuals positive at each time point. Between and within-group comparisons of medians at each time point were done with Mann–Whitney and Wilcoxon rank tests, respectively. Differences were considered significant if $p \leq 0.05$.

3. Results

3.1. Cellular immune responses

3.1.1. Cytotoxic T lymphocyte response

Although CTL reactivity was generally low, differences in the proportion of CTL-positive animals were found among the study groups (Fig. 1). In the control group, no evidence of CTL response was detected in the pre-challenge period (Fig. 1A) and the percentage of positive animals in the post-challenge period was similar to that found in the groups receiving B7. In the gag–env immunized group, CTL activity was found after the MVA boost (week 12), resulting in over 50% positive animals; but the highest activity was reached at week 20, with more than 80% positive animals (Fig. 1B). In this group, statistical differences were found significant between the pre-challenge (weeks 0 and 7) and the post-challenge (week 20) periods. In the gag–env–CD80 group, CTL-positive animals appeared at week 7 as a result of plasmid immunization in 25% of the animals (Fig. 1C). This low proportion was maintained until week 16. By the end of the experimental period CTL activity decreased (12.5% positive animals) but did not disappear. In the gag–env–CD80–CD86 group, the highest proportion of CTL-positive animals was reached early after immunization (week 7, 63%), significantly increased compared to week 0. The number of CTL-positive animals decreased thereafter, especially post-challenge, until disappearing by the end of the experimental period (Fig. 1D).

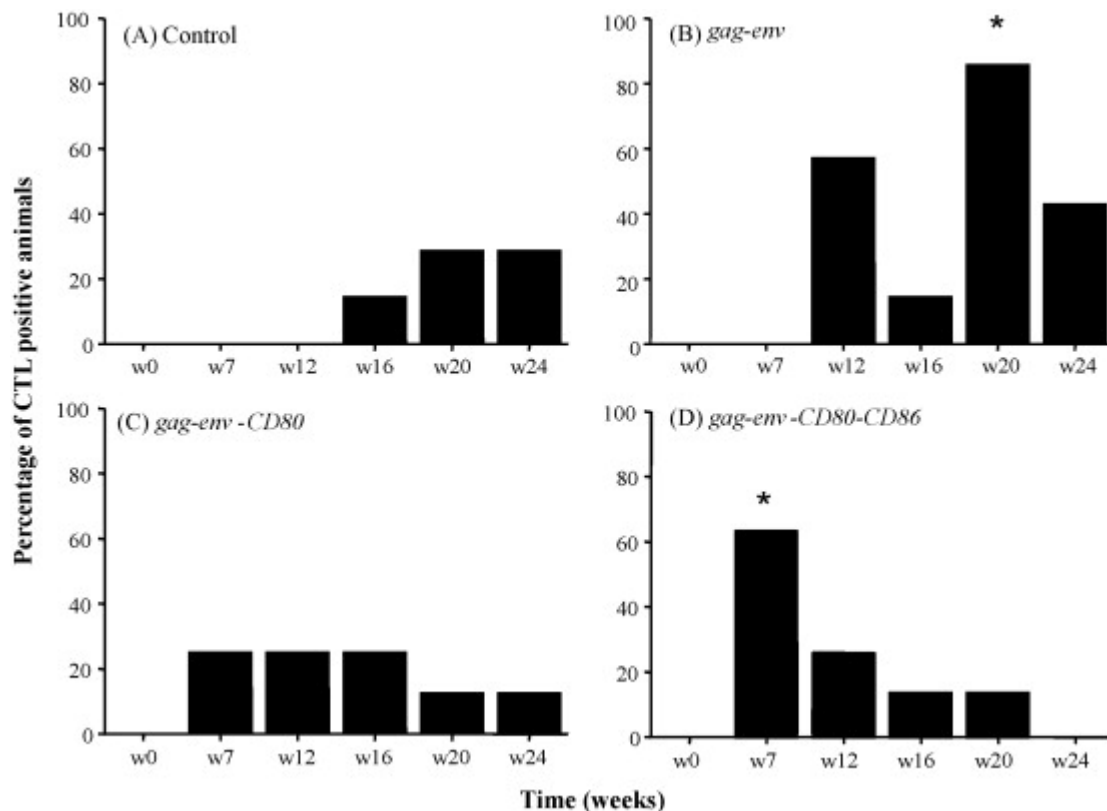


Fig. 1.

CTL responses. The percentage of CTL-positive animals in the control and immunization groups is shown. (*) Statistically significant differences between weeks of the experimental period in the percentage of CTL-positive animals according to Fisher's Exact test within each study group: in the *gag-env-CD80-CD86* group, the percentage in week 7 was significantly higher ($p = 0.025$) than in week 0; and in the *gag-env* group the percentage in week 20 was higher than in weeks 0, 7 ($p = 0.004$ in both cases) and 16 ($p = 0.029$).

A comparative analysis between groups revealed a significantly increased proportion of CTL-positive animals at week 7 in the *gag-env-CD80-CD86* group (63%) compared to control and *gag-env* groups (both 0%). In contrast, both B7 immunized groups had significantly decreased numbers of CTL-positive animals compared to the *gag-env* group (12.5% in each B7 group vs. 85.7% in the *gag-env* group) at week 20.

Overall these results indicate that inclusion of B7 genes in *gag-env* immunization results in a transient (week 7) increase in the CTL response pre-challenge (this increase being significant when both B7 genes are included in the inoculum) but a decreased CTL response post-challenge.

3.1.2. T lymphocyte proliferative responses

3.1.2.1. Effect of plasmid immunization

PBMC from the animals under study were stimulated with GAG protein (p14, p17 or p25) and S.I. of proliferative responses determined (Fig. 2). Comparatively, the p14 and p25 proteins frequently induced the lowest and the highest S.I., respectively. Within-group analysis revealed that both groups immunized with B7 genes had a significantly increased ($S.I. \geq 3$) early (week 7) proliferative response against all of the GAG proteins. Also, between group comparisons (Fig. 2) indicated that the early response (week 7) was significantly increased in the CD80/CD86 group compared to the control group when using p17 as stimulatory protein; and in both B7 groups compared to the *gag-env* group using either p14 or p25.

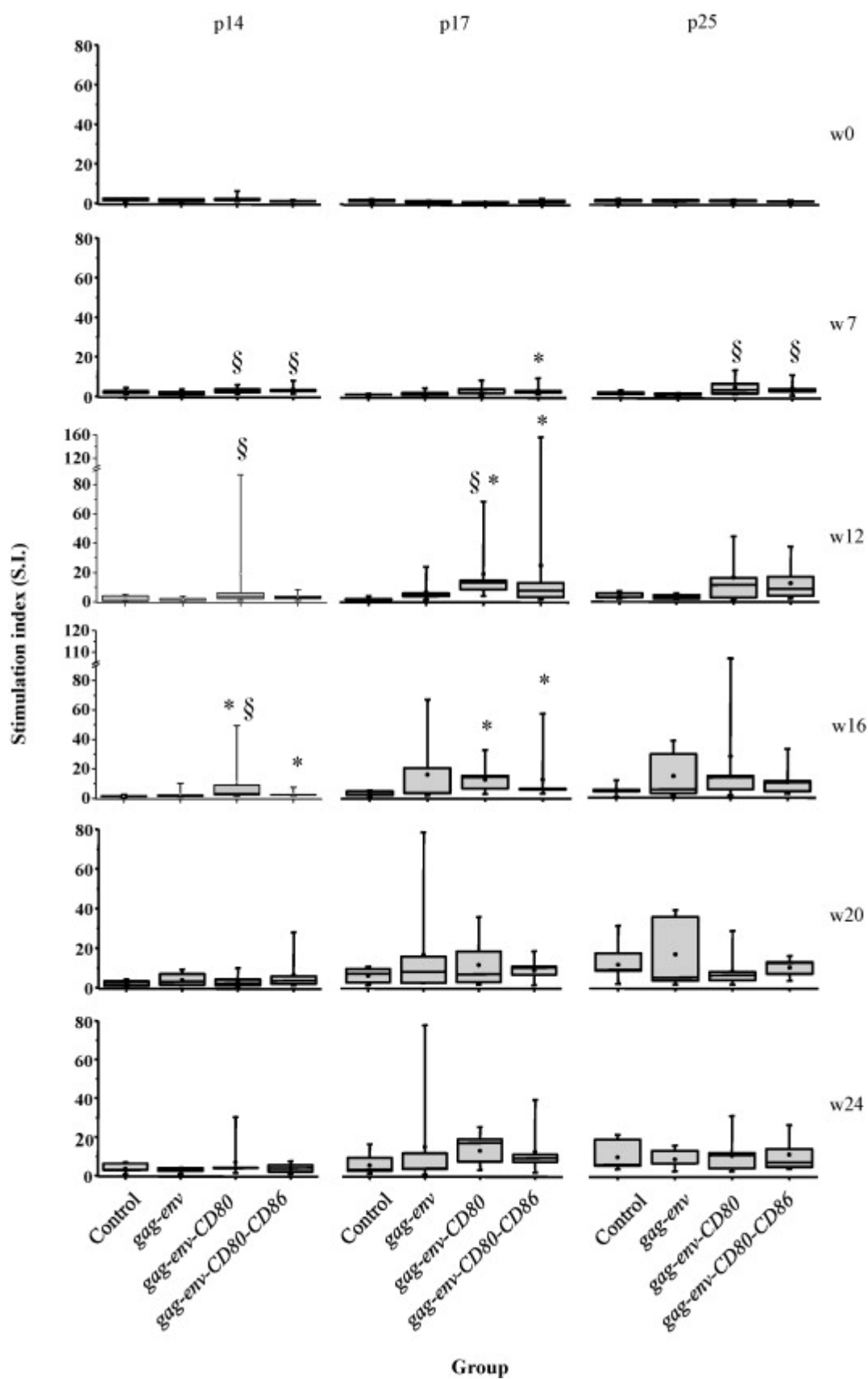


Fig. 2.

T cell proliferative responses. The antigen stimulation indices (S.I.) in the different groups throughout the study period (weeks 0–24) are shown, using as stimulatory antigen p14, p17 or p25 GAG proteins. Results are expressed as box plots with the median of the group shown as a line and the interquartile range represented by the box. (*) Shows values significantly higher ($p < 0.05$) than the control group according to a Mann–Whitney

test, found: for p14, gag-env-CD80 and gag-env-CD80-CD86 groups at week 16; for p17, gag-env-CD80 group at weeks 12 and 16; and gag-env-CD80-CD86 group at weeks 7, 12 and 16. (§) Significantly higher ($p < 0.05$) than gag-env group, found: for p14, gag-env-CD80 group at weeks 7, 12 and 16; and gag-env-CD80-CD86 group at week 7; for p17, gag-env-CD80 group at week 12; for p25, gag-env-CD80 and gag-env-CD80-CD86 groups at week 7. See text for intra-group differences between time points.

3.1.2.2. Effect of MVA booster immunization

In both B7 immunized groups, boosting with recombinant MVA (week 10) resulted, by week 12, in a further significant increase of the proliferative response compared to plasmid immunization (weeks 0 and 7) responses using either p17 or p25 as stimulatory antigens. Furthermore, within the gag-env group in the absence of B7 genes in the inoculum, the proliferative response was significantly increased only after the MVA booster immunization (using p17 and p25 protein for stimulation, significance not indicated on the graph). Analysis between groups (Fig. 2) revealed that by week 12 animals receiving B7 genes had a significantly increased response compared to the control group using p17 for stimulation. Finally, the gag-env-CD80 group had a significantly higher response than the gag-env group using either p14 or p17.

3.1.2.3. Effect of VMV challenge

By week 16, both groups receiving B7 maintained the proliferative responses observed before challenge (week 12) and had significantly increased responses compared to the control group using p14 or p17 (Fig. 2). However, the control group had a significantly increased response when comparing in this group the post-challenge and the pre-challenge periods (using p17 and p25 proteins, weeks 20 and 24, significance not indicated in the figure). Later post-challenge time points (weeks 20 and 24) showed similar responses in the different study groups.

Overall, immunization including B7 triggered an increased proliferative response to viral antigen before and early after challenge compared to gag-env and control groups. After challenge, the proliferative response was maintained at moderate levels in all groups until the end of the experiment.

3.2. Specific antibody production

Antibody titre determinations using a whole virus ELISA revealed that VMV antibodies were not significantly produced before challenge in any of the groups (Fig. 3A). After challenge, groups receiving CD80 (alone) or CD80 and CD86 had the highest antibody production compared to the control group, being significantly higher at weeks 16, 20 and 24 for the gag-env-CD80 group; and at weeks 16 and 20 for the gag-env-CD80-CD86 group. Compared with the gag-env group, both B7 groups also had significantly higher antibody titres at week 16; and the gag-env-CD80 group had increased titres at week 24. In all groups, the antibody titre increased towards the end of the experimental period. These results were confirmed with a commercial VMV antibody ELISA test (Elitest). More than 50% of the vaccinated animals became seropositive (O.D. ratios >1) in this assay after challenge (by week 16) in each of the immunized groups, whereas the control group reached this percentage later on (week 20).

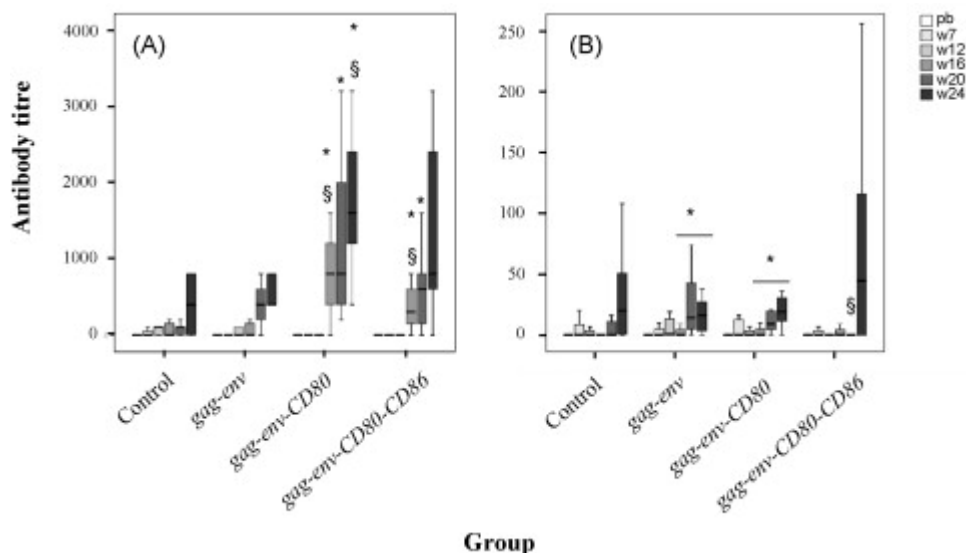


Fig. 3.

Antibody production against VMV. Panel A shows antibody titres against VMV as detected by serial two-fold dilutions of sera from sheep tested on a whole VMV ELISA at different time points. Sera were not considered positive unless titre was >100. The results are represented by box plots indicating the median value and the interquartile range. (*) Titre greater than the control group ($p < 0.05$, group gag-env-CD80 at weeks 16, 20 and 24; and group gag-env-CD80-CD86 at weeks 16 and 20); (\$) titre greater than group gag-env ($p < 0.05$, group gag-env-CD80 at weeks 16 and 24; group gag-env-CD80-CD86 at week 16), using Mann-Whitney tests. Panel B shows neutralization titres against VMV. Different dilutions of the sheep serum were incubated with the virus. Neutralizing antibody titres were calculated as the reciprocal of the serum dilution that caused neutralization in 50% of viral infectivity in the inoculated cultures. Median and interquartile range are represented in the box plot. (*) Statistical differences between pre-challenge and post-challenge periods within groups ($p = 0.046$ for gag-env; $p = 0.043$ for gag-env-CD80) by Wilcoxon tests. (\$) Statistical differences between gag-env-CD80-CD86 vs. gag-env and gag-env-CD80 groups at week 20 ($p = 0.05$ and $p = 0.038$, respectively), using Mann-Whitney tests.

WB determinations using whole virus antigen (not shown) provided information on antibody specificity. Antibodies produced soon after challenge (by week 16) in both B7 immunization groups had anti-ENV precursor (gp150) and anti-GAG (p25) specificities. Furthermore, most of the B7 vaccinated animals produced anti-ENV gp44 transmembrane (TM) protein antibodies, but only at late stages after challenge (week 24). Antibodies found by WB in the gag-env group soon after challenge (weeks 16 and 20) were only against the ENV precursor, anti-GAG antibodies appearing later on in this group (at week 24). The control group presented antibodies only at week 24, having then anti-ENV and anti-GAG specificities.

Regarding NtAb production (Fig. 3B), the gag-env-CD80 and the gag-env vaccinated animals showed significantly increased titres in the post-challenge (weeks 16, 20 and 24) compared to the pre-challenge period (week 12). Interestingly, amongst the immunized groups, NtAb titre was the lowest (at week 20) in the gag-env-CD80-CD86 group, as only one of the eight animals within this group had NtAb at this time point. However, this difference was not maintained, as four of the eight animals became NtAb positive at week 24. Three of these four NtAb positive animals, reached then high NtAb titres and were provirus positive. On the other hand, among the four NtAb negative animals of this group, only one was provirus positive (see Section 3.3).

Overall, no significant antibody response was detected in groups immunized with B7 genes (CD80 alone or combined with CD86) and the other groups upon immunization and booster. Although after challenge the B7 groups had a higher antibody response compared to the control group, significant NtAb production was detected the latest (week 24) in the gag-env-CD80-CD86 immunized animals and only in 50% of them.

3.3. Proviral load after challenge

Proviral load was measured in blood and other tissues by quantitative real-time PCR before (to verify the PCR-negative status) and after EV1 challenge (done at week 12) and the proportion of provirus positive animals determined. All the animals were free of provirus before challenge.

In blood, just after challenge (week 16), all groups showed at least one provirus positive animal. During the post-challenge period, provirus was detected at least once in all the animals vaccinated with gag-env or gag-env-CD80 and the majority of animals in the control group. Significantly, only 50% of the animals were provirus positive in the gag-env-CD80-CD86 group (Table 2), a proportion that was significantly lower than in the other immunized groups, gag-env-CD80, gag-env and the control group.

Table 2.

Percentage of animals with virus (PCR+) in blood and/or tissues after challenge with VMV.

Group (number of animals)	Blood ^a	Other tissues			Total (animals with virus in blood and/or other tissues)
		Lung ^b	Mediastinal lymph node	Total	
<i>gag-env-CD80</i> (<i>n</i> = 8)	100	37.5	50	71.4	100
<i>gag-env-CD80-CD86</i> (<i>n</i> = 8)	50 ^c	25	12.5	25	50 ^d
<i>gag-env</i> (<i>n</i> = 7)	100	42.8	0 ^e	42.8	100
Control (<i>n</i> = 7)	85.7	57.1	28.6	57.1	100

a Infection was considered to be present when at least one of the three time points analyzed after challenge with VMV (weeks 16, 20 and 24) showed a positive reaction.

b Infection was considered to be present when at least one of the four different lung pieces analyzed showed a positive reaction.

c In blood, statistical analysis showed that the group receiving both B7 genes (*gag-env-CD80-CD86*) had fewer provirus positive animals than the group without costimulatory molecules (*gag-env*) ($p = 0.05$) as well as the group with *CD80* alone ($p = 0.038$).

d An animal was considered positive for infection when any positive reaction was found in blood and/or in the different tissues. The animals receiving *gag-env-CD80-CD86* had a decreased percentage of infected animals when compared to the rest of the groups: *gag-env-CD80* group ($p = 0.038$) and *gag-env* and control groups ($p = 0.05$) using Fisher Exact test.

e In tissues, statistical differences were found between the *gag-env* group and *gag-env-CD80* in the mediastinal lymph node ($p = 0.05$).

Proviral load was also determined in other tissues (lung and MLN). Viral detection in one of the four analyzed pieces of the lung or in the MLN was considered indicative of infection. Surprisingly, no provirus was detected in the MLN of the gag-env group, but when both target tissues were analyzed together, the gag-env-CD80-CD86 group showed again the lowest infection level with only 25% of animals provirus positive (Table 2).

When analysing the three sampled tissues together (blood, lung and MLN), the group including both B7 genes in the inoculum (*gag-env-CD80-CD86*) showed a significantly decreased proportion of provirus positive animals compared with the other groups (50% vs. 100%).

Proviral load was highly variable among the infected animals in all the groups (10–700 copies). There were no significant differences between groups in the proviral load amongst the infected animals.

3.4. Post-mortem examination

Lung and MLN were examined at necropsy. All the animals showed macroscopically healthy lungs. Upon microscopic examination, most of them, including those of the gag-env and control groups, showed bronchial associated lymphoid tissue hyperplasia in the absence of gross clinical signs such as congestion and/or oedema. Follicular hyperplasia and interstitial pneumonia in lungs and lymphoid follicle reactivity and cortical hyperplasia in MLN were scored jointly, as described elsewhere [16], to determine the overall histological changes. The results showed that groups immunized with B7 genes had a significantly higher score index than the control group (Fig. 4). This augmented score was due to an increased frequency of animals in these groups that presented: (a) follicular hyperplasia in lung (all the B7 immunized animals vs. 70% of the gag-env and 56% of control animals); (b) a mild interstitial pneumonia (62% vs. 50% and 0% of the animals in the B7 groups compared to gag-env and control groups, respectively); and (c) follicular reactivity in MLN (all the B7 immunized animals vs. 50% of the gag-env and 70% of control animals). Interestingly, perivascular infiltrates were absent from lungs of both B7-immunized groups (except in 1 of the 16 animals) but generally present in the control and gag-env groups.

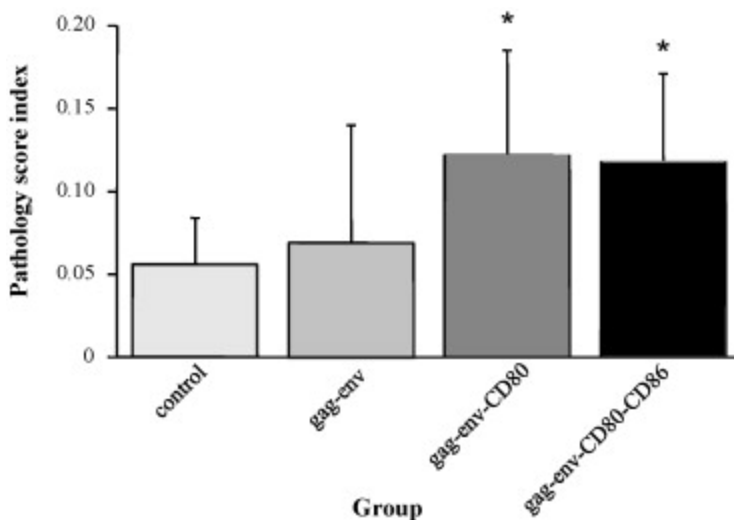


Fig. 4.

Post-mortem tissue examination. The mean pathology score \pm standard error of the group in lung and mediastinal lymph node is shown. (*) Pathology score greater than the control group ($p = 0.021$ for gag-env-CD80; $p = 0.009$ for gag-env-CD80-CD86) using a Mann-Whitney test.

Altogether, these results demonstrate an increased lymphoid tissue reaction and the absence of gross clinical signs in target organs (mild lung interstitial pneumonia and follicular hyperplasia in lung and MLN), upon B7 adjuvant immunization-intratracheal challenge with VMV.

4. Discussion

Knowing that CD80 RNA levels in VMV infections are downregulated in clinically affected animals [32], we investigated in this study whether the use of ovine B7 genes in immunizations with VMV gag and env genes enhanced specific immune responses in sheep and conferred significant protection after challenge in terms of viral infection and/or immune enhancement. For this, a combined vaccination strategy against VMV in sheep was applied, consisting of PMED recombinant plasmid delivery [14] and [15] followed by a subcutaneous boost with recombinant MVA [15] and VMV challenge. This allowed the study of cellular and antibody mediated immune responses along the experimental period and determination of proviral load as well as investigation of histological changes at necropsy.

Early CTL/Th1 responses after vaccination, previously studied in infections by HIV, SIV, CAEV and EIAV, have been associated with protection in individuals challenged with the virus [41], [42], [43], [44] and [45]. Here, the earliest immune responses observed upon vaccination were mainly cellular, not antibody mediated. An early CTL response was produced as a result of plasmid immunization (by week 7) in both B7 immunization groups, being then significantly increased in the group receiving both B7 genes. However, the response decreased after MVA boost (by week 12) and further decreased in both B7 immunization groups, until disappearing in the group receiving both CD80 and CD86 after challenge. In contrast, the proportion of precursor CTL-positive animals had a tendency to increase in the control group in the post-challenge period. In a previous vaccination-challenge study, using the same gag-env immunization approach without including B7 genes in the inoculum, no CTL-positive animals were found by week 7, rather CTL responses appeared later, after MVA boosting, and became significantly higher compared to unvaccinated controls by week 20 ([15] and this study). Also, with a gag-env mucosal immunization approach, a very limited CTL response was obtained upon immunization and challenge [16]. Altogether, these observations strongly suggest that inclusion of B7, and particularly both B7 genes (CD80 and CD86) in the immunization inoculum leads to an early (week 7) significant production of CTL precursors. Likely, these cells underwent clonal expansion and re-distribution in the organism (week 12) and were maintained in a low proportion in blood after virus clearance (weeks 16, 20 and 24). The enhancement of CTL responses when incorporating CD86 into the vaccine is in agreement with a series of previous HIV studies in mice [46], [47] and [48] where inclusion of CD80 in the inoculum did not generate CTL responses. However, the relative role of both CD80 and CD86 molecules remains controversial, as there are studies in mice which either propose the enhancement of both cellular and humoral responses when using CD86 [49] or describe the enhancement of CTL responses when incorporating CD80 rather than CD86 [50].

Like CTL production, significantly increased proliferative responses against a GAG protein were found as early as week 7 (upon plasmid immunization), but only in the group immunized with both B7 genes, compared to unvaccinated controls. Upon MVA boosting (week 10), proliferative responses became significantly increased in both groups receiving B7 genes. Using the same immunization protocol in sheep immunized with gag-env (without including B7), the proliferative response against a GAG protein was also found by weeks 7 and 12 [15]. Following challenge (performed at week 12), proliferative responses against GAG proteins were significantly increased compared to controls (week 16) in both studies ([15] and this study) and in work involving sheep mucosal gag-env immunization [16]. Similarly to this work, a gag-pol plasmid immunization study in chimpanzees demonstrated that inclusion of CD86 gene in the inoculum augmented the viral-antigen-specific lymphoproliferative responses [51]. Overall, this study demonstrates that inclusion of B7 genes (especially if both genes are combined) in gag-env immunization results in increased early lymphocyte proliferative responses, and strongly suggests that some of these lymphocytes may be involved in CTL activation. The group receiving both B7 genes had a decreased proportion of provirus positive animals and a significantly increased early CTL and proliferative responses after plasmid immunization, leading to a high clonal expansion and strong memory cell production by the time of challenge. Similarly, in an in vitro study a role for CD86 rather than CD80 in suppression of HIV replication has been linked to enhanced CD8+ cell activity [52].

Antibody (IgG), as detected by ELISA, was not produced upon plasmid or MVA immunization in any of the immunization groups, which is in agreement with previous VMV plasmid immunization studies in sheep [14] and [16], and with work in mice on immunization with HIV plasmids in the presence of CD80 or CD86 genes [49]. Just after challenge (week 16) and until the end of the experimental period, groups receiving B7 genes produced both anti-ENV and anti-GAG antibodies, as revealed by WB, whereas the gag-env group had anti-ENV but a very low production of anti-GAG antibodies at weeks 16 and 20 and the control group

did not show either anti-ENV or anti-GAG antibodies until week 20. In natural infections, anti-ENV and GAG p25 antibodies are usually produced the earliest [53] although there can be a delay in the production of anti-ENV antibodies [54]. In this work, the delay was restricted to TM-ENV antibodies, likely because some TM epitopes are less exposed. The presence of ENV- and particularly GAG-specific antibodies in both of the B7 immunized groups early after challenge could be due to increased costimulatory signals and pre-activation of the B cells as a result of immunization (weeks 7 and 12) in these groups.

NtAb production was very limited in this study, increasing in all the groups towards the end of the experimental period. The delay in NtAb production observed in the group receiving both B7 genes (CD80 and CD86) may be related to a decreased viral exposure in this group after challenge, with only 50% vs. 100% of provirus positive animals. Also the probability of finding NtAb was decreased in provirus-free animals of this immunization group by week 24. A similar association between the lack of NtAb production and decreased viral load/exposure has been found in vaccinated animals of a previous VMV env-based immunization-challenge study [14]. Furthermore, NtAb have been found in VMV vaccinated animals that become infected after challenge [20]. NtAb were detected post-challenge in this study, and not in our previous immunization study performed under the same experimental conditions but in the absence of B7 adjuvants [15]. This was most likely due to differences in the sensitivity of the neutralization assay used in both studies, as here its readout was viral replication (using a β -galactosidase construct) whilst Niesalla et al. [15] detected syncytium formation.

An early, strong NtAb response has been linked to reduced CNS pathology after infection of sheep with VMV [55], and neutralization escape mutants do arise in persistently infected animals [56] and [57]. However, there is mounting evidence that neutralization by antibody may not be important in controlling SRLV infections [58] and [59]. Likely, some of the NtAb detected in vitro are inefficient in controlling the infection in vivo. On the other hand, in the immunodeficiency virus field, antibodies functioning in antibody-dependent cellular cytotoxicity (ADCC) responses may be important in controlling virus (SHIV) replication [58]. As VMV is primarily cell associated in vivo, ADCC may be a particularly important antibody effector function. However, IgG2 antibody involved in ADCC, is not induced after VMV infection [36] and [60] and induction of this isotype by vaccination may provide a significant mode of protection. Future studies to determine which antibody isotypes and which cellular and cytokine profiles are induced by prime-boost vaccination regimens are needed.

Significantly, PMED immunization using VMV gag and env genes, together with B7 genes combined, triggered significantly increased early specific CTL and proliferative responses, leading to the presence of precursor and effector memory T cells. These cells could be present in the lung and the MLN, having local follicular hyperplasia (both) and a mild interstitial pneumonia (lung) upon intratracheal challenge, as observed at necropsy. An increased reaction score in target tissues (lesion) was also observed in a previous study on immunization with the combination of the gag and env genes [15]. However, all the animals receiving B7 had overall mild lesions/inflammatory signs. There was no disappearance of epithelial tissue and the lesions (presence of lymphocytes, follicular hyperplasia, etc.) would be those expected from a transient antigen exposure. None of the animals had clinical signs, macroscopic or strong microscopic lesions, that might be considered irreversible. These tissue changes, observed at the third month post-challenge (this work and previous studies; [15] and [16]), might disappear when prolonging the post-challenge period for about one year, as observed in previous work employing the same viral dose and strain for challenge [14].

The fate of lesions may be linked to the presence/absence of infection. In this regard, a significant proportion (50%) of animals in the group receiving both B7 genes was provirus negative while showing mild lesions, but in these animals, the immunization could have just delayed the appearance of infection. If so, virus-associated clinical disease would be expected to take more than one year to appear [14], taking into account the challenge dose used and the prolonged period often required in lentiviral infections for target organs to present severe lesions. Alternatively, these animals could have been really protected from the viral infection. In this case, a progressive disappearance of the interstitial pneumonia and of follicular hyperplasia would be expected as antigen exposure would be missing. In any case, the immune response was able to either eliminate the virus or delay the establishment of infection in these animals during the experimental period. To distinguish between these alternatives, cell and cytokine profile determinations in target tissues/organs would be relevant again in longer term post-challenge studies. These will also help to understand why the sensitization before challenge eventually enhances or stops the lesion in these animals.

In conclusion, in VMV gag-env vaccination, the use of B7 genes particularly in combination (CD80 and CD86), induces early increased cellular responses (CTL and proliferative) before challenge as well as antibody responses to viral antigens soon after challenge and enhances protective effects against early infection while triggering an immune reaction of lymphoid tissue in challenged-target organs. Overall, the use of B7 genes combined results in decreased infection, but new strategies need to be explored in order to further increase the proportion of provirus-free healthy animals in long term immunization-challenge approaches and determine the final outcome and profile of the inflammatory reactions in target organs and related tissues.

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